REPORT DOCUMENTATION PAGE			Form Approved OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE August 2003	3. REPORT TYPE AND DATES COVERED Annual (1 Aug 2002 – 31 Jul 2003)	

4. TITLE AND SUBTITLE Roles of Steroid Receptor Coactivators in Breast Cancer	5. FUNDING NUMBERS DAMD17-99-1-9430

6. AUTHOR(S)

Meei-Huey Jeng, Ph.D.

20040602 018

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Indiana University Indianapolis, IN 46202-5167 E-Mail: mjeng@iupui.edu	8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012	10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited	12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

The purpose of this study is to determine the roles of steroid receptor coactivators in breast cancer. The scope of the research is assessing the expression of SRC-1 family members (SRC-1, TIF2, and AIB1) during breast cancer progression using breast cancer tissue and adjacent normal tissue obtained from Indiana University Tissue Procurement Facility. In addition, functions of these coactivators have been studies during this report period. We have constructed adenovirus expressing these three coactivators. We found that AIB1 protein was overexpressed in human breast cancer specimens, as compared to adjacent normal breast tissue. In addition, increased expression of AIB1 in human breast cancer cells stimulated the S phase entry during cell cycle progression, indicating the involvement of AIB1 in cell cycle control and cell growth of human breast cancer cells. Furthermore, overexpression of AIB1 dramatically increased the phosphorylation of Akt and Akt kinase activity in human breast cancer cells. These studies provide important insights how AIB1 works in human breast cancer cells to promote breast cancer progression.

14. SUBJECT TERMS breast cancer, endocrinology, adenovirus, AIB1, SRC-1, coactivators	15. NUMBER OF PAGES 14
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

AD_____

Award Number: DAMD17-99-1-9430

TITLE: Roles of Steroid Receptor Coactivators in Breast Cancer

PRINCIPAL INVESTIGATOR: Meei-Huey Jeng, Ph.D.

CONTRACTING ORGANIZATION: Indiana University
Indianapolis, IN 46202-5167

REPORT DATE: August 2003

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

Table of Contents

Introduction

The subject of the current studies is to determine the expression and functions of steroid receptor coactivator-1 (SRC-1) family members in breast cancer. Previously, we have evaluated the expression of SRC-1 family members in human breast tumors and found that AIB1 was overexpressed in many breast tumor specimens. The scope of the research is assessing the functions of SRC1 family members during breast cancer progression. We will also assess the functions of SRC-1 family members by constructing adenovirus expressing coactivators and determine the molecular actions of these coactivators in human breast cancer cells.

Body

Task 1: Completed. Collection of mammary gland samples has been completed.

Task 2: Initiated. RNA has been extracted and will be analyzed for coactivator expression using Northern blot and RT-PCR techniques.

Task 3: Eliminated per contract agreement.

Task 4: Completed. We have treated animals with estrogen benzoate or tamoxifen, and mammary gland samples have been collected.

Task 5: Initiated; to examine the expression of coactivators in mammary gland upon hormone and antihormone treatment.

Task 6: Completed. We have completed the collection and embedding of human breast tumor specimens.

Task 7: Completed. See results presented at previous annual report. The immunohistochemical staining of SRC-1 and AIB1 in human breast tumors has been completed. AIB1 was overexpressed in human breast tumors, after comparing 122 clinical specimens. A manuscript is in preparation to document the staining data. We are facing background problems using TIF2/GRIP1 antibody for immunohistochemical staining. We are still trying to improve this background problem.

Task 8: Completed. See results presented at previous annual report.

Task 9: Completed. We have done preliminary staining of human breast cancer MCF-7 cells using anti-SRC-1 and anti-AIB1 antibodies. Nuclear staining of SRC-1 and AIB1 were observed. To a less degree, the cytoplasmic staining was observed. This is in contrast to the tumor staining we have performed. In several cases, we observed the cytoplasmic staining of SRC-1 and AIB1 in tumor cells in human breast tumors. We are not certain whether the hormonal level in patients or cells will influence the localization of SRC-1 or AIB1 in cells.

Task 10: Completed. To examine the expression of p160 coactivators in human breast cancer cell lines, we have done Western blot analysis for SRC-1, GRIP1/TIF2, and AIB1 in several human breast cancer cell lines. Both ERα-positive (MCF-7, T47D, and ZR-75-1) and ERα-negative

4

(HCC1937, MCF10A, MDA-MB-231, MDA-MB-435S, BT-20, and SKBR) cells express all three coactivators. MCF-7 cells expressed a much higher level of AIB1 among all the cell lines tested.

Task 11: Completed. We have successfully constructed the sense construct of the adenovirus expressing AIB1, GRIP1/TIF2 and SRC-1. These viruses are tagged with GFP in order to monitor the infection and expression efficiency of this virus. The control virus AdGFP has also been constructed.

Task 12: Completed. We have completed the testing for adenoviruses constructed in tissue culture cells. We have performed Western blot analysis and are able to show that AdGFP-AIB1 express functional AIB1. Preliminary data indicate that overexpression of AIB1 increased the S phase of cell cycle in T47D cells (Figure 1) and MCF-7 cells (data not shown). Addition of PI3K/Akt inhibitor LY294002 abolished the AIB1 enhanced S phase entry (Figure 2). Infection of human breast cancer cells with AIB1 increased Akt phosphorylation (Figure 3) and Akt kinase activity (Figure 4), suggesting the involvement of Akt signaling in mediating AIB1 regulated cell cycle progression. Furthermore, AIB1 was able to interact with c-Src in immunoprecipitation experiment (Figure 5), an important adaptor protein for growth factor signaling. In transfection experiment, AIB1 and c-Src was able to synergistically activate ER transactivation function (Figure 6). These data suggest that cross-talk of AIB1 signaling with growth factor signaling is playing an important role in mediating AIB1 actions in human breast cancer cells.

Task 13: Completed. Large scale preparation of adenoviruses has been completed through CsCl banding.

Task 14: We have initiated this task and have infused the purified adenoviruses into rat mammary gland for testing the effects of AIB1 on modulating ER signaling pathway in situ.

Task 15: Initiated. We have started collecting samples for histology examination and in situ β-galactosidase reporter activity in order to complete this task.

Key Research Accomplishments

-We have demonstrated that AIB1 protein was altered in many breast cancer tumors.

- We have successfully constructed adenovirus expressing AIB1, SRC-1 and GRIP1 for further

testing of the functions of these coactivators.

- We have demonstrated that AIB1 over-expression could increase the S phase of the cell cycle.

Addition of LY294002 was able to abolish the AIB1 stimulated increase of S phase entry.

-We found that AIB1 was able to interact with c-Src adaptor protein. Transfection of AIB1 with c-

Src enhanced the ER transactivation function synergistically in human breast cancer cells.

-Infection of human breast cancer cells with adenovirus expressing AIB1 increased the

phosphorylation of Akt and Akt kinase activity. LY294002 was able to abolish the AIB1

stimulated increase of S phase entry, suggesting the involvement of growth factor signaling

pathways in mediating AIB1 action in human breast cancer cells.

Reportable Outcomes

Abstracts:

1. Long, X., Cai, A., Vieth, E., Quilliam, L.A., and **Jeng, M.-H.** Interaction of c-Src with Steroid Receptor Coactivators. Keystone Symposium D4: Nuclear Receptor Superfamily, 2002. Late break-through oral presentation.

2. **Jeng, M.-H.,** Zhang, Q.-H., Long, X., Goulet, R., Sledge, G., Li, L., and Quilliam L. Functions of Estrogen Receptor Coregulators in Breast Cancer. DOD Era of Hope Meeting, 2002.

Conclusions

During this funding period, we have completed many of the tasks, especially the evaluation of AIB1 actions in human breast cancer cells. We have demonstrated that AIB1 can activate Akt signaling pathway. The interaction of AIB1 with c-Src signaling also provides important insights of the molecular actions of AIB1 in human breast cancer cells. The generation of AdGFP-AIB1 will provide us a valuable tool to assess the functional role of AIB1 in ER transactivation function in mammary gland and in regulating breast cancer cell proliferation. Our current studies suggest that overexpression of AIB1 protein may contribute to increased S phase and subsequently increased cell proliferation in human breast epithelial cells.

References

1. Shim, W.-S., DiRenzo, J., DeCaprio, J. A., Santen, R. J., Brown, M. J., Jeng, M.-H. Segregation of steroid receptor coactivator-1 from steroid receptors in mammary epithelium. Proceedings of the National Academy of Sciences 96(1): 208-213, 1999.

2. Jeng, M.-H., Kao, C., Sivaraman, L., Krnacik, S., Chung, L. W. K., Medina, D., Conneely, O. M., and O'Malley, B. W. Reconstitution of estrogen dependent transcriptional activation of an adenoviral target gene in select regions of the rat mammary gland. Endocrinology 139(6): 2916-2925, 1998.
3. .Zhang, Q.-H., Chang, L.-Y., Vieth, E., Stallcup, M.R., Edwards, D.P., Cheng, L., Goulet, R.J., and Jeng, M.-H. Over-expression of Several Nuclear Receptor Coactivator Proteins in Human Breast Carcinoma. 83rd Annual Meeting of the Endocrine Society, June, 2001.

4. Long, X., Cai, A., Vieth, E., Quilliam, L.A., and **Jeng, M.-H.** Interaction of c-Src with Steroid Receptor Coactivators. Keystone Symposium D4: Nuclear Receptor Superfamily, 2002. Late break-through oral presentation.

5. **Jeng, M.-H.,** Zhang, Q.-H., Long, X., Goulet, R., Sledge, G., Li, L., and Quilliam L. Functions of Estrogen Receptor Coregulators in Breast Cancer. DOD Era of Hope Meeting, 2002.

Appendices

Six figures and two abstracts.

Figure1 . AIB1 stimulated S phase entry in T47D cells

Legend:
- Ad GFP
- Ad GFP AIB1

X-axis: % of Cells in S Phase (0, 5, 10, 15, 20, 25, 30, 35, 40)
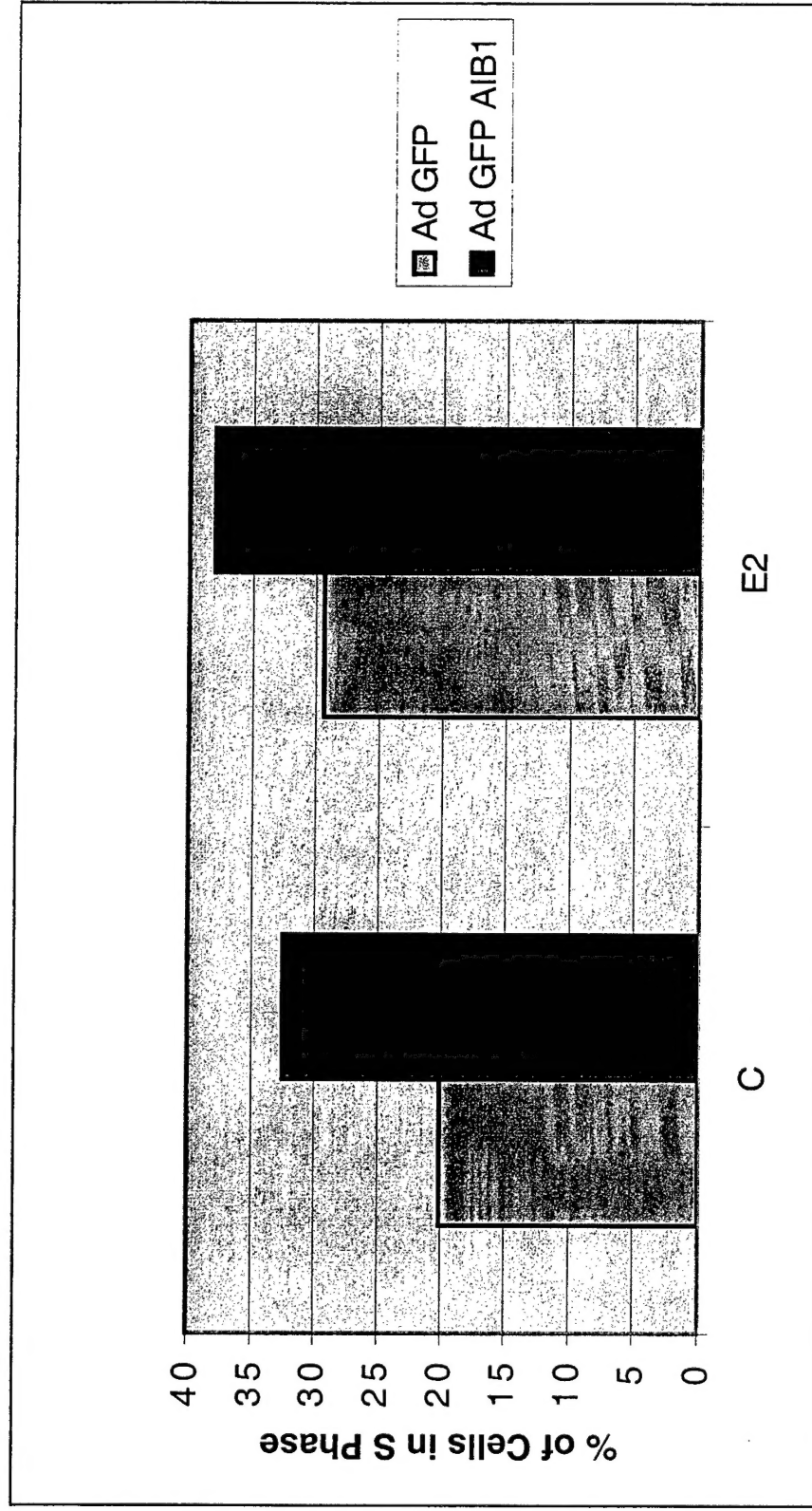
Categories: C, E2

Figure 2. LY294002 abolished the AIB1-stimulated increase of S phase entry in T47D cells
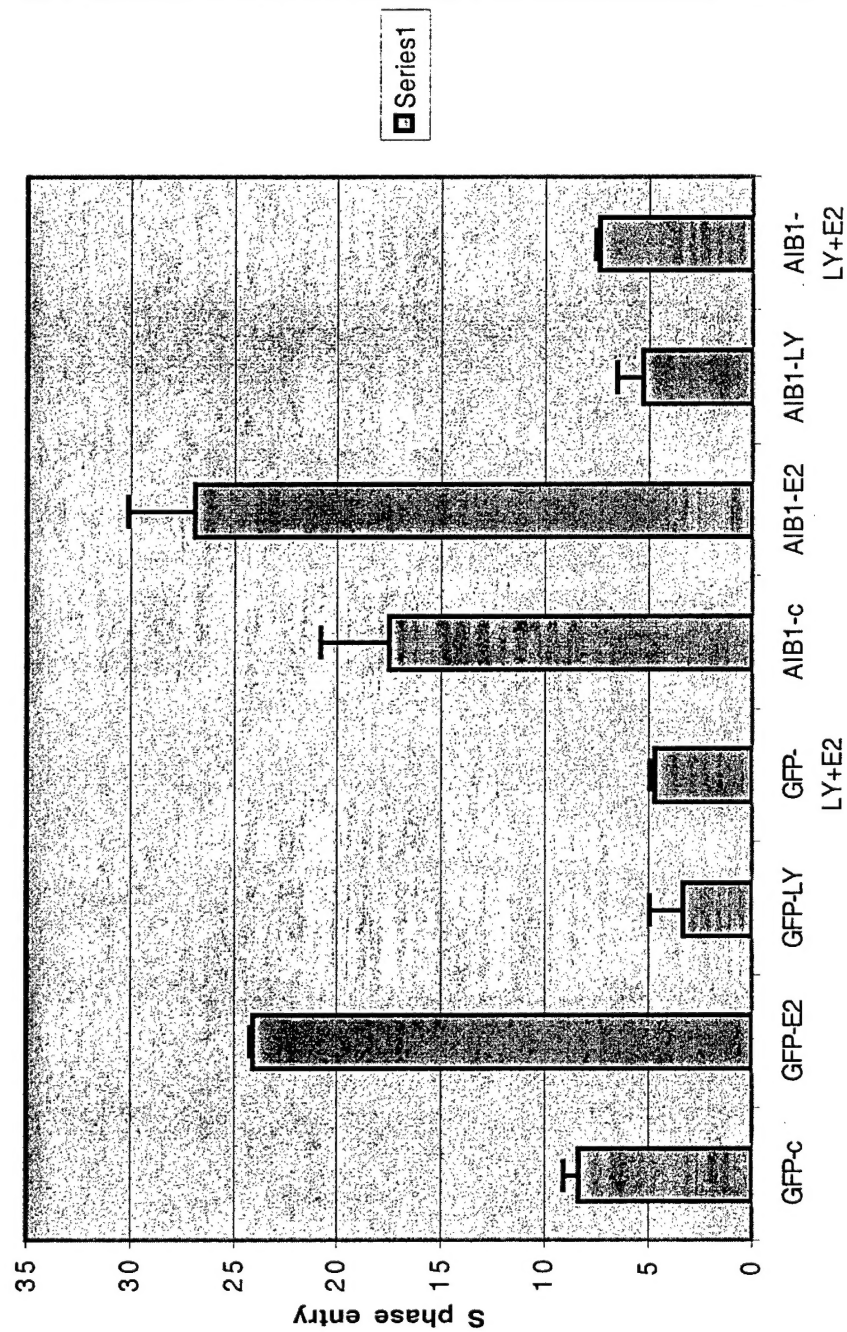
Figure 3. Ad-AIB1 overexpression increased Akt phosphorylation in MCF-7 cells
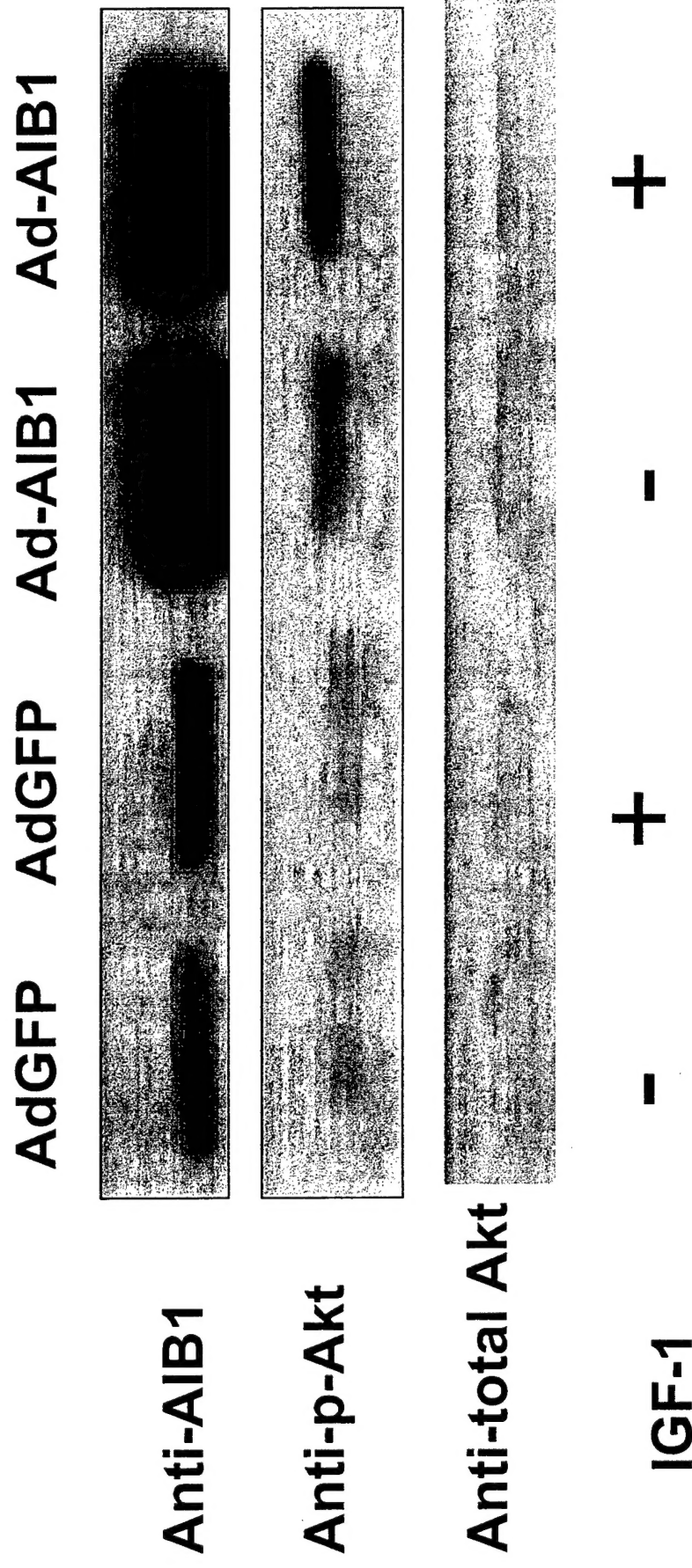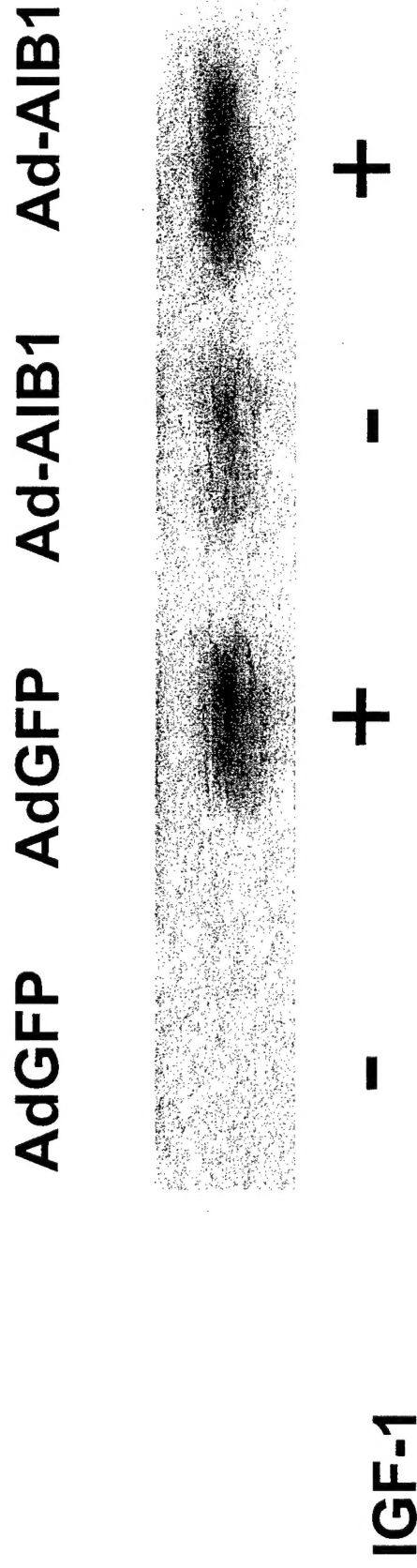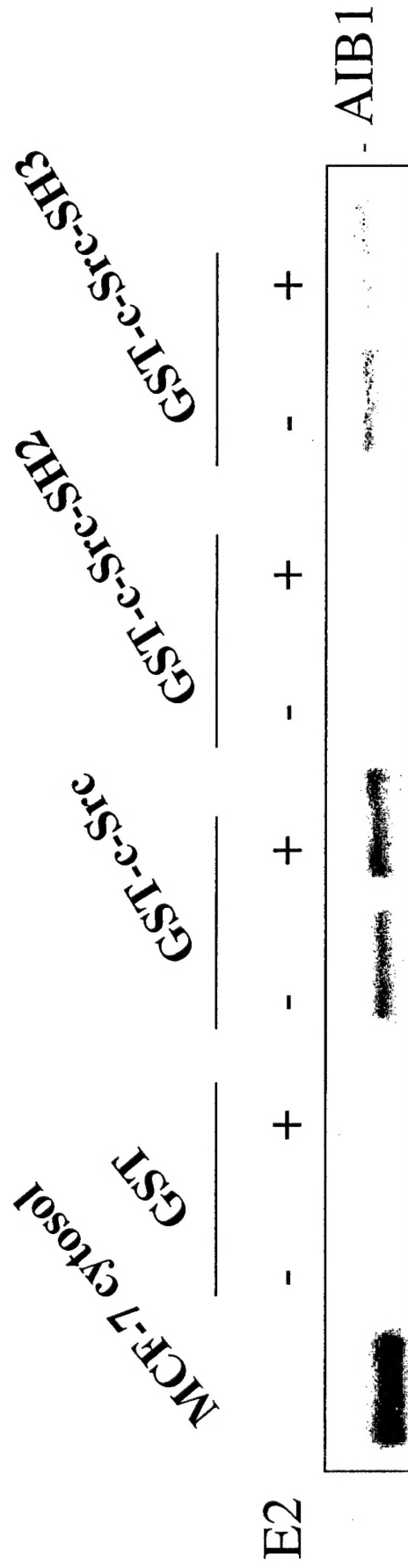
Figure 4. Ad-AIB1 overexpression increases Akt kinase activity in MCF-7 cells

	AdGFP	AdGFP	Ad-AIB1	Ad-AIB1
IGF-1	−	+	−	+

Use histone H2B
as the substrate in Akt kinase assay

Figure 5. Interaction of AIB1 with c-Src in MCF-7 cells in immunoprecipitation experiment

Western blot:αAIB1 Ab

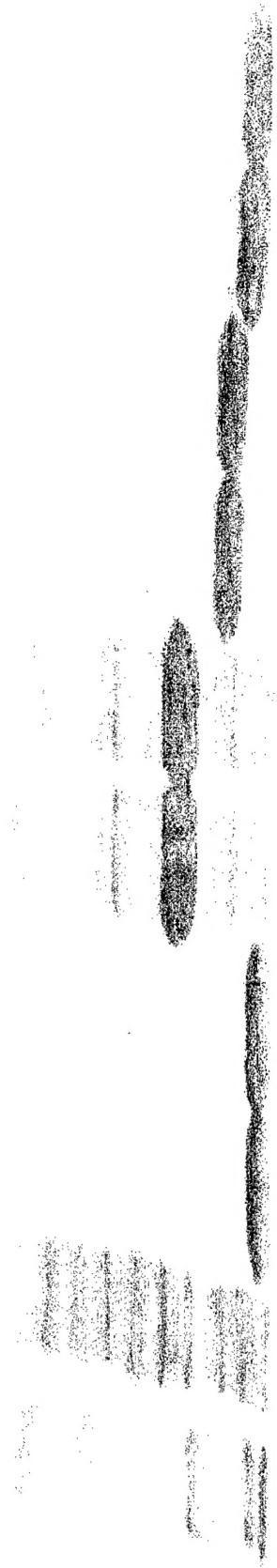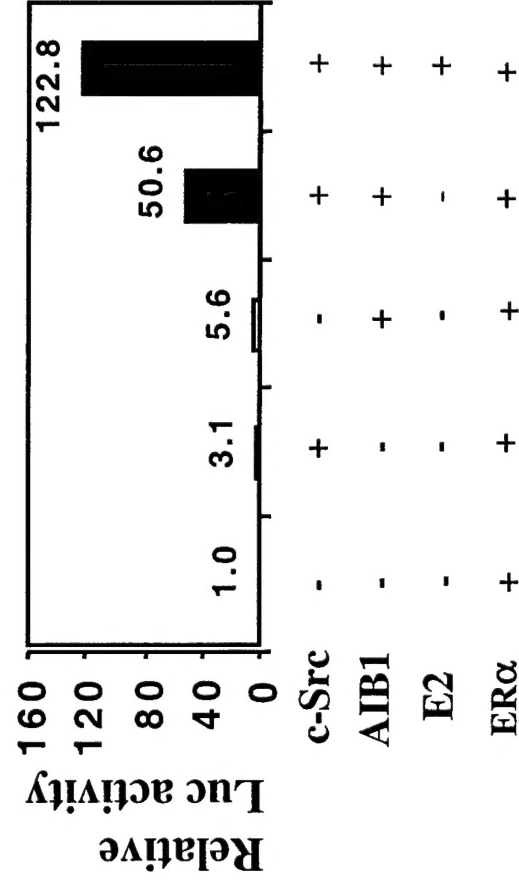|	MCF-7 cytosol	GST	GST-c-Src	GST-c-Src-SH2	GST-c-Src-SH3	
E2	-	+	-	+	-	+	-	+	-

AIB1

Ponceau S Staining

Figure 6. Synergism of AIB1 and c-Src on ER transactivation function in transfection experiment

Interaction of c-Src with Steroid Receptor Coactivators

Xinghua Long[1,3], Aijun Cai[1,3], Edyta Vieth[1,3], Lawrence A. Quilliam[2,3], and Meei-Huey Jeng[1,3]
[1]*Departments of Medicine and* [2]*Biochemistry & Molecular Biology, Indiana University School of Medicine and* [3]*the Walther Oncology Center, Indianapolis, Indiana, USA, 46202*

Estrogen receptor α (ERα) regulates transcription of target genes by recruiting coactivator proteins, including three p160 family members, steroid receptor coactivator 1 (SRC-1), transcriptional mediators/intermediary factor 2 (TIF2/GRIP1) and amplified in breast cancer 1 (AIB1/RAC3/pCIP). Increasing evidence has suggested that cross-talk of steroid receptors with growth factor signaling components may play important roles in regulating gene expression mediated by steroid receptors. In this report, we examined the roles of steroid receptor coactivators in cross-talk of growth factor signaling with steroid receptors. We found that several adapter proteins made up of Src homology (SH) domains, including c-Src, Grb2, and PLCγ interacted with ERα in GST-pulldown assays. Interestingly, these same adapter proteins could also interact with SRC-1, GRIP1, and AIB1. SH2 domain of c-Src was required for its interaction with ERα. However, SH3 domain was required for c-Src interaction with p160 coactivators. Interactions of c-Src with ERα and p160 coactivators were further confirmed by co-immunoprecipitation experiments. Protein Kinase assay indicated that c-Src phosphorylated AIB1, and the phosphorylation site was located at the C-terminal region of the AIB1 containing the multiple LXXLL motifs important for interaction with steroid receptors. This study suggest that coactivators can serve as the convergent point linking growth factor signaling to steroid receptor and that phosphorylation is likely a critical event for this type of gene regulation. Supported by NCI-RO1-CA82565, DOD and Walther Oncology Institute. MHJ is supported by DOD Breast Cancer Research Program Career Development Award.

FUNCTIONS OF ESTROGEN RECEPTOR COREGULATORS IN BREAST CANCER

Meei-Huey Jeng, Qinghui Zhang, Xinghua Long, Robert Goulet, George Sledge, Liang Cheng, Lang Li, and Lawrence Quilliam

Departments of Medicine, Pathology, Surgery, and Biochemistry, Indiana University School of Medicine, Indianapolis, IN 46202

mjeng@iupui.edu

Estrogen signaling components have been shown to be important in breast cancer progression. These include estrogen receptor alpha (ER) and its coregulators such as steroid receptor coactivator 1 (SRC-1) family (p160) members, SRC-1, SRC-2/TIF2/GRIP1, and SRC-3/AIB1. These ER coregulators can regulate ER transactivation function in a ligand dependent fashion. Increasing evidence suggests that the cross-talk of steroid receptors with growth factor signaling may have an important role in regulating gene expression mediated by steroid receptors. This report examines the expression of SRC-1 family members in human breast tumors and the role of ER coregulators in cross-talk between growth factors and ER signaling. We analyzed 122 cases of breast carcinoma obtained from the IU Tissue Procurement Facility, 79 cases of breast carcinoma in tissue array slides obtained from NCI, and 29 cases of breast carcinoma in commercial breast sausage slides (containing an additional 15 cases of fibroadenoma, and 15 cases of normal breast tissue). We found that AIB1 protein was overexpressed (p-value < 0.001). When normal breast epithelial MCF10A cells and breast cancer MCF-7 cells were infected with an adenovirus expressing AIB1, the S phase was increased dramatically, suggesting that AIB1 was involved in cell cycle regulation. Furthermore, several growth factor signaling adapter proteins made up of Src homology (SH) domains, including c-Src, Grb2 and PLCg, interacted with ER in GST-pulldown assays. Interestingly, these same adapter proteins could also interact with SRC-1, GRIP1, and AIB1. The SH2 domain of c-Src was required for interaction with ER. The SH3 domain of c-Src was required for interaction with p160 coactivators. Interactions of c-Src with ER and p160 coactivators were further confirmed by co-immunoprecipitation. Protein kinase assay indicated that c-Src phosphorylated AIB1. The phosphorylation site was located at the C-terminal region of AIB1 containing the multiple LXXLL motifs important for interaction with steroid receptors. Our data suggest that coactivators can be the convergent point linking growth factor signaling to ER and that phosphorylation is likely a critical event for this type of gene regulation in breast cancer cells.